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PRINCIPAL INVESTIGATOR: Elizabeth Wayner, Ph.D.
                             Richard Tamura, Ph.D.

CONTRACTING ORGANIZATION: Seattle Biomedical Research Institute
                                 Seattle, Washington 98109-1651

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6. AUTHOR(S)
Elizabeth Wayner, Ph.D.
Richard Tamura, Ph.D.

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Seattle Biomedical Research Institute
Seattle, Washington 98109-1651

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13. ABSTRACT *(Maximum 200)*
We are interested in studying the role of integrins in regulating cell proliferation in normal and transformed mammary epithelial cells. During this past year, we found that signals transduced by clustering of integrins were capable of initiating cell proliferation of Go-synchronized normal human mammary epithelial cells (HMEC). Taken together with last year's studies using RGD peptides, it appears that multiple integrins participate in the regulation of cell proliferation of both normal and transformed cells. Surprisingly, fibronectin did not promote the growth of Go-synchronized HMEC while both type I collagen and to a lesser extent laminin-5 had growth promoting effects. This may reflect differences in signaling among the integrins or possibly mechanisms that are active during different stages of the cell cycle. The normal human mammary epithelial cell line MCF-12A and HMEC have similar integrin profiles and similar adhesion capabilities to laminin5 and fibronectin. However, MCF-12A cells express the breast cancer antigen CA15-3 while HMEC do not. This suggests that MCF-12A cells resemble luminal epithelia while HMEC resemble basal cells. Additional studies are planned to define the signaling pathways utilized by integrins to regulate the proliferation of normal and transformed mammary epithelial cells.

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Richard Tanner   10/28/97
PI - Signature   Date

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I. INTRODUCTION

A. Nature of the Problem
Breast cancer is the leading cause of cancer-related death among women in this country and the incidence of breast cancer among young women is rising Although early detection techniques (mammography) are available, early detection has not led to a decline in mortality rates and cannot be used to prevent or treat breast cancer. Developing new strategies to improve breast cancer survival will depend upon a greater understanding of the pathogenesis of breast cancer and the events which lead to neoplastic transformation of breast epithelial cells.

Cancer cells differ from normal cells in that they undergo uncontrolled cell growth and acquire the abilities to invade adjacent tissues, enter the circulatory system, and "home" to sites distal from the primary tumor (metastasis). An important aspect of tumor invasion and metastasis involves the adhesive interactions of tumor cells with other cells or the extracellular matrix. Many of these adhesive interactions are mediated by the integrin family of cell adhesion receptors (reviewed by Hynes, 1992). Integrins provide not only a structural means of cell anchorage but also a means of transmitting signals regulating gene expression and protein function (Clark and Brugge, 1995). It is possible that extracellular matrix proteins regulate normal cell growth by transmitting signals to the interior via specific integrin receptors. In transformed cells, such signaling pathways might be absent or dysfunctional thereby conferring unresponsiveness to normal growth constraints. **Our efforts are focused to understand how integrins participate in the regulation of cell division in normal breast cells and to determine how breast cancer cells escape these regulatory pathways.**

B. Background of Previous Work

1. Integrins. Integrins are transmembrane glycoproteins comprised of two non-covalently associated subunits (α and β) that mediate both cell-cell and cell-substrate adhesion. Integrin receptors bind extracellular matrix (ECM) and plasma proteins, non-integrin adhesion receptors and other integrins (reviewed by Hemler, 1990: Hynes, 1992). In addition to their role as primary mediators of cell adhesion, it is now become clear that integrins are also capable of transducing signals to the cell interior (Hynes, 1992, Ginsberg et al, 1992, Juliano, 1993, Juliano, 1994, Clark and Brugge, 1995. Tyrosine phosphorylation appears to be a key aspect of integrin mediated signal transduction and a tyrosine kinase (p125 FAK) has been identified which localizes to focal adhesions when cells adhere to an ECM protein-coated surface (Schaller et al, 1992). In addition, evidence is accumulating which suggests that integrin-mediated signaling events can induce gene expression (Yurochko et al, 1992) and affect transit through the cell cycle (Matsuyama et al, 1989, Symington, B.E., 1992, Mortarini et al, 1992). Many of the ECM proteins that serve as ligands for integrins have been identified and include: fibronectin (FN), vitronectin (VN), laminins, and collagens. Some integrins can interact with more than one ligand (e.g. $\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha 3\beta 1$) while several ECM proteins are recognized by more than

one integrin. For example, FN has been shown to interact with multiple integrin receptors ($\alpha5\beta1$, $\alpha4\beta1$, $\alpha v\beta3$, $\alpha v\beta6$, $\alpha v\beta1$, $\alpha3\beta1$)

2. Cell cycle Regulation. The initiation of cell division and transitions between different stages of the cell cycle involves signals which activate the association of specific protein complexes (cell cycle dependent kinases (cdk) with the regulatory cyclins). At each checkpoint certain cyclins and CDKs form active complexes which phosphorylate and thereby activate specific proteins necessary for DNA replication (G1/S), mitosis, and cytokinesis (G2/M). Recently, it has been shown that the activity of cyclin/CDK complexes is regulated by a family of CDK inhibitor proteins or CDIs (p16, p21, p27) that bind to and inactivate the CDKs (Hunter and Pines, 1994). **The original purpose of this project was to determine how signals transduced via integrin receptors, especially a5b1, regulated the formation of cdk/cyclin complexes in neoplastic and normal breast cells.**

Initial results suggested that engagement of a fibronectin receptor, the integrin $\alpha5\beta1$, with its fibronectin peptide ligand, GRGDS, affected cdc2/cyclin A complexes in partially transformed but not fully transformed epithelial cells (Symington, B.E., 1995). Last year we expanded Dr. Symington's experiments with GRGDS peptides to normal mammary epithelial cells and 5 additional breast cancer cell lines. We did not observe concomitant increases in both cdc2 kinase and cyclin A associated kinase activities. There also did not appear to be any correlations between these cell cycle kinase activites and either the tumorigenic potential of the cells or the profile of RGD binding integrins. In addition, immunohistochemistry of both normal and tumor breast tissue indicated that $\alpha5\beta1$ was not expressed by either normal or neoplastic mammary epithelial cells. The predominant integrin expressed by both normal and tumor cells was $\alpha3\beta1$. Thus, as indicated from the 1996 progress report and the comments of the reviewers, it was appropriate to depart from the original Statement of Work on investigating the role of the integrin $\alpha5\beta1$ in the regulation of cell proliferation of normal mammary epithelial cells and possible defects in this process in cancer cells.

3. Change in Statement of Work

Our revised Statement of Work and Specific Aims, which are intended to maintian the spirit of the original Statement of Work, are:

Statement of Work:
The purpose of this project is to determine the role of integrins in regulating the proliferation of normal and neoplastic mammary epithelial cells.

Specific Aims:
Aim 1: Determine whether all integrins or a subset of integrins are involved in regulating normal mammary epithelial cell proliferation.

Aim 2: Determine the mechanisms of integrin-mediated cell cycle regulation.

Aim 3. Examine carcinoma cells for possible defects in these mechanisms.

C. Purpose of the Present Work

Most of the work for the past year has focused on Aim 1 of the revised Statement of Work. We examined the role of integrins in normal human mammary epithelial cells (HMEC) cultured in serum-free conditions by looking at the effects of specific ECM proteins on cell growth and through clustering of specific integrins with monoclonal antibodies.

II. BODY

A. Methods

1. Patient Samples

Tissue specimens were obtained from lumpectomy and mastectomy tissues provided by Dr. Ron Tickman who is a staff pathologist in the Laboratory of Pathology, Swedish Hospital Medical Center, Seattle, WA and used for immunohistochemistry and the isolation of breast cells (BC). These samples were removed from patients undergoing surgery at Swedish Hospital solely for diagnostic or therapeutic purposes and would otherwise be discarded. Information identifying the patients was withheld in order to comply with the requirements to obtain exemption status from complete IRB review. However, we have access to information concerning the patient's age and sex, tumor histology, estrogen and progesterone receptor status, and the presence and location of metastases. This will enable me to correlate tumor type, malignancy, integrin expression and response to a5b1 ligation. Although no patient samples will be excluded on the basis of race, age, sex, religion, or ethnic background, the low incidence of male breast cancer will preclude collection of a large number of samples from men. I have received approval for the use of these tissues from the IRB at SBRI.

2. Monoclonal and Polyclonal Antibodies

Anti-integrin Monoclonal Antibodies. Several monoclonal antibodies (Mabs) directed to a variety of integrin receptors expressed by normal and neoplastic epithelial cells have been produced, previously (Wayner and Carter, 1987; Wayner et al, 1988; Carter et al., 1990a, 1990b; Wayner et al, 1991). Many of these Mabs perturb integrin function and will be used to determine how normal and neoplastic breast cells interact with FN or GRGDS peptide. These Mabs include the two used by Dr. Symington to ligate $\alpha 5$ or $\beta 1$ (P1D6 (anti- $\alpha 5$) and P4C10 (anti- $\beta 1$) as well as inhibitory antibodies P1H5 (anti-$\alpha 2$), P1B5 ($\alpha 3$), P4C2 ($\alpha 4$), P5H9 ($\alpha v\beta 5$), LM609 ($\alpha v\beta 3$, courtesy of Dr. David Cheresh, The Scripps Research Institute, La Jolla, CA), GoH3 (anti-$\alpha 6$, Pharmingen, San Diego, CA), and AA3 (anti-$\beta 4$, courtesy of Dr. Vito Quaranta, The Scripps Research Institute, La Jolla, CA).

Antibodies Directed to Human Cell Cycle Proteins. Monoclonal or polyclonal antibodies directed to human CDKs (cdc2, cdk2-cdk5), cyclins (A, B1, D1-D3, E), CDIs (p16, p21, p27), and tumor supressor proteins (Rb and p53) were obtained from Pharmingen (San Diego, CA). The Mab to breast cancer antigen CA15-3 was obtained from Chemicon Intl. (Temecula, CA). E-cadherin was detected with Mab HECD-1 (courtesy of M. Takeichi).

3. Breast Cell Isolation and Culture

Normal human BC was obtained from Clonetics Corp (San Diego, CA, cat. # CC-0228) or from reduction mammoplasty tissue supplied by Dr. Ron Tickman. Breast epithelial cells were isolated according to published protocols (Taylor-Papadimitriou and Stampfer, 1992; Blaschke, R.J. et al., 1994, Bergstraesser L.M and Weitzman, S.A., 1993). Briefly, the samples were minced and enzymatically digested to isolate mammary glands and single cells. After an initial plating in serum-containing medium, cells were cultured in serum-free mammary epithelial growth medium (MEGM) from Clonetics (cat. #CC-3051). A number of breast carcinoma cell lines (see Table I) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured as recommended by the ATCC.

4. Flow Cytometry Analysis

Flow cytometry analysis was used to identify the integrin receptors expressed by cultured normal and transformed BC lines. Cells were incubated with anti-integrin Mabs (10 mg/ml or culture supernatant) for 30 min in suspension in FACS buffer (HBSS supplemented with 1% goat serum and 0.02% sodium azide). Cells were then washed and incubated with affinity purified FITC-conjugated goat anti-mouse, 2 mg/ml (Southern Biotechnology, Birmingham, AL). The appropriate isotype matched controls were included with each sample. Cells were analyzed by forward light scatter (linear) versus green fluorescence (log). Flow cytometric analysis of stained cells were performed on either an Coulter EPICS C System or a Becton Dickinson FACScan equipped with an argon laser. At least 5,000 events were analyzed for each anti-integrin antibody and compared to a matched isotype control.

5. Immunohistochemistry

Normal and tumor breast tissue were obtained from Dr. Ron Tickman, embedded in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN), and frozen on a block of dry ice. Tissue sections (6-10 microns) were cut using an IEC cryostat. Sections were analyzed for expression of integrins and extracellular matrix proteins using monoclonal antibodies as described by Hoffstrom and Wayner (1994).

6. Kinase Assay

The *in vitro* kinase assay was performed essentially as described (Symington, B.E., 1992). Briefly, normal or breast cancer cell lines were incubated for 2 h in culture media

alone or in media containing GRGDS (or GRGES) peptides (10 μg per 5 x 10^5 cells). Cell lysates were prepared and reacted with anti-cdc2, anti-cdk2, anti-cyclin A, anti-cyclin E, or normal rabbit serum followed by protein A-agarose beads. Protein A beads were washed and resuspended in kinase buffer (20 mM Tris pH 6.8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 30 mM ATP, 10 mCi of [γ-^{32}P]-ATP (3000 Ci/mmol; Amersham Corp) with or without 1 μg histone H1 per reaction tube and incubated at 37° C for 30 min prior to solubilization in SDS-PAGE sample buffer and electrophoresis on 12% polyacrylamide slab gels. Gels were stained with Coomasie brilliant blue, dried, and exposed to film.

7. Integrin clustering

Cells were first synchronized by culturing in the absence of any serum or exogenously added growth factors for 36 to 48 h. Monoclonal antibodies to specific integrin subunits were bound to the cells for 30 minutes at 4°C in growth factor-free media. Unbound primary antibodies were washed from the cells. The bound monoclonals were then cross-linked with the appropriate polyclonal secondary (rabbit anti-mouse or rabbit anti-rat) in media containing reduced levels of growth factors (1/10 to 1/100 of the normal media) and the cells were placed back at 37° C. Maximal clustering occurred approximately 30 minutes after addition of the cross-linking secondary antibodies and could often be detected within 15 minutes. Cells were lysed by scraping the cells into lysis buffer (1% NP-40 in 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml leupeptin, and 10 mg/ml aprotinin). The protein concentration of each lysate was determined using the BCA protein assay kit (Pierce, Rockford,IL). Fifty or 100 micrograms of total protein were seperated by SDS-PAGE on 6% polyacrylamide gels, transferred to PVDF membranes (Biorad, Hercules, CA) and probed with antibodies to the retinoblastoma protein. Bound antibodies were detected using the colorimetric system of alkaline phosphatase conjugated to goat anti-mouse antibodies along with NBT and BCIP as substrates (Promega, Madison, WI).

8. Adhesion and proliferation assays.

96 well microtiter plates were coated with purifed ECM proteins overnight at 4° C. Fibronectin was obtained from Life Technologies (Gaithersburg, MD). The source of type I collagen was Vitrogen 100 from Collagen Biomaterials (Palo Alto, CA). Conditioned media from normal human foreskin keratinocytes was used as a crude source for laminin5. Laminin5 was captured onto the microtiter wells by first coating the wells with the laminin5 monoclonal antibody C2-5 (generously provided by Susanna Gill and Dr. Bill Carter, Fred Hutchinson Cancer Research Center). After coating with ECM proteins, the wells were washed with PBS and blocked with 1% BSA in DMEM.

For cell adhesion studies, cells were resuspended in blocking solution and 50,000 to 100,000 cells were added to each well. Cells were allowed to adhere for 30 minutes at 37°C, washed with Dulbecco's PBS, fixed with 3% paraformaldehyde in PBS, and stained

with 0.5 % crystal violet. Wells were washed in tap water and the bound dye was eluted with 1% SDS. Absorbance of each well was read at 540 nm in a plate reader.

For cell proliferation studies, cells were first synchronized by culture under growth factor free conditions for 36 to 48 hours. Cells were harvested by trypsinization and the trypsin was neutralized with soybean trypsin inhibitor. Ten thousand cells were added to each well in either growth factor-free or normal media. After 3 to 5 days of culture, the attached cells were fixed and stained as described above for the adhesion studies.

B. Results and Discussion

In last year's report we indicated that $\alpha 5 \beta 1$ was not expressed by either normal or cancerous mammary epithelial cells *in vivo* and therefore could not play a significant role in the regulation of cell proliferation. At the time of the report, we were in the process of analyzing two additional breast cancer cell lines, Hs578T (partially transformed) and MDA-MB-361 (fully transformed). Incubation with RGD peptides did not effect either cdc2 or cyclinA-associated kinase activity in Hs578T cells and increased cdc2 kinase activity but had no effect on cyclin A-associated kinase activity in MDA-MB-361 cells. The results of our experiments on the modulation of cyclinA/cdc2 kinase activity by RGD peptides is summarized in Table I. These data suggest that multiple integrins were involved in regulating cell cycle kinases in both normal and tumorigenic mammary epithelial cells. Since increases in cdc2 kinase activity did not correlate with increases in cyclin A-associate kinase activity, these results also seem to indicate that integrins can modulate multiple cdk/cyclin complexes.

A number of experimental approaches were utilized in an attempt to investigate the role of integrins in regulating proliferation of normal mammary epithelial cells:

1. ECM protein stimulation of cell proliferation
2. ECM protein stimulation of cell cycle kinase activities
3. Modulation of cell cycle kinase activities in response to integrin clustering in cell cultures synchronized via growth factor starvation
4. Phosphorylation of Rb in response to integrin clustering on synchronized cells.

1. ECM protein stimulation of cell proliferation. Of the three ECM proteins tested, type I collagen had the strongest, dose-dependent stimulation of normal HMEC cell proliferation (Fig 1). Laminin5 may have had a slight effect although a dose dependence over 3 orders of magnitude was not observed. Surprisingly, fibronectin, starting at 30 mg/ml coating concentration, did not stimulate cell proliferation under these conditions. This stimulation of cell growth occurred in the absence of exogenously added growth factors. These results suggest that those integrins that mediate cell adhesion to type I collagen and possibly to laminin5 may be more important than receptors for fibronectin in regulating cell proliferation. Integrins that have been characterized as collagen receptors include $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, and $\alpha 3 \beta 1$. HMEC cells express both $\alpha 2 \beta 1$ and $\alpha 3 \beta 1$. We have not tested for $\alpha 1 \beta 1$ expression. Both laminin5 receptors, $\alpha 3 \beta 1$ and $\alpha 6 \beta 4$, are expressed by

HMEC cells. Alternatively, the collagen used for this experiment may contain bound growth factors that are not present in either the laminin5 or the fibronectin.

2. ECM protein stimulation of cell cycle kinase activities. We did not observe any integrin-mediated stimulation of cdc2 kinase, cdk2 kinase, cyclin A-associated kinase, or cyclin D-associated kinase activities in HMEC cells adhered to type I collagen, fibronectin, PronectinF (a polymer containing RGD sequences), human foreskin keratinocyte conditioned medium (crude source for laminin5 and fibronectin), and poly-L-Lysine for 30 minutes or 2 hours. Based on our results in the RGD peptide experiments, we expected to see an effect during this time period. However, it may be that we should have expanded these studies to longer time points to see an effect.

3. Modulation of cell cycle kinase activities in response to integrin clustering in cell cultures synchronized via growth factor starvation. In HMEC cells that were synchronized by growth factor starvation, cdc2 kinase activity was induced between 8 and 24 h after re-addition of growth factors (Fig 2A). Under these conditions, clustering of beta1 integrins may have helped to keep the culture synchronous during their transit through the cell cycle as evidenced by the apparant sharper rise of cdc2 kinase activity and fall back to pre-induction levels by 36 h (Fig 2B).

4. Phosphorylation of Rb in response to integrin clustering on synchronized cells. Rather than continue with kinase assays to evaluate effects on the cell cycle, we decided to use the phosphorylation of the retinoblastoma (Rb) protein as our indicator of cell cycle progression. Rb is phosphorylated by a number of cyclin dependent kinases and appears to be a crucial event in the passage of cells through the restriction point in G1. Phosphorylated forms of Rb were detected by Western blot analysis and were distinguised from unphosphorylated forms by an increase in the apparent molecular weight of the protein. In normal mammary epithelial cells, clustering of the b1, a3, b4, or av integrins induced Rb phosphorylation beginning at about 8 h after clustering with phosphorylated Rb being the predominant form at 16 h (Fig. 3). This stimulation required the presence of growth factors. If growth factors were completely eliminated from the media during the experiment, we did not observe phosphorylation of Rb. These results indicate that a variety of integrins may be involved in regulating cell cycle progression, including those which do not bind RGD (i.e. a6b4) and that the integrins are working in conjunction with gowth factors as has been reported for other cell types.

Characterization of MCF12A cells. During this past year, most of our studies have utilized normal primary mammary epithelial cells obtained from Clonetics Corp., San Diego, CA. While working with primary cells should make these studies more relevant to the in vivo situation, the inherent difficulties of working with primary cells are slow growth rates and limited cultivation due to terminal differentiation and/or senescence. This has hampered our abilitites to conduct some experiments in a timely fashion, especially those that required significant expansion of cell numbers. MCF10A is a cell line that has been used as a model for normal mammary epithelial cells. Unfortunately, the American Type Culture Collection (ATCC) has encountered difficulties maintaining

this cell line and we been unable to receive these cells that we requested in May of 1996. Recently, we received the cell line MCF-12A from the ATCC. This cell line has been described to be "substantially normal, but immortalized" by a number of criteria (Pauley et al., 1993). These cells differ significantly from normal HMEC in their expression of the breast cancer antigen CA15-3 (Fig 4). We showed in last year's report, that CA15-3 was expressed on the apical surface of normal luminal mammary epithelium. Thus, the MCF-12A cell line appears to be a good model cell line for studying cell proliferation regulation of luminal mammary epithelial cells. While it is possible that CA15-3 expression is somehow related to the establishment of these cells as immortal, it is probably more a reflection on the origin of these cells as luminal. MCF-12A exhibits similar cell adhesion characteristics as normal HMEC to the ECM proteins laminin5 and fibronectin (Fig 5). The reduced level of adhesion to fibronectin by MCF-12A cells may be due tor lower levels of the integrins $\alpha 5$ and $\alpha 4$. MCF-12A cells also express the integrins $\alpha 3$, $\alpha 6$, $\beta 4$, $\alpha 2$, $\alpha v\beta 5$, but not $\alpha v\beta 3$, at levels similar to normal HMEC. Phosphorylation of Rb occurs between 8 and 24 h after growth factor stimulation of proliferation, similar to HMEC (Fig. 6). However, we have not observed any significant changes in Rb phosphorylation as a result of integrin clustering. Part of the problem is that we have not yet found a concentration of growth factors that does not significantly stimulate Rb phosphorylation in the absence of integrin clustering.

We recently obtained the cell line MDA-MB-435 because this cell line has been described as being highly metastatic. These cells differ from both normal HMEC and MCF12-A cells in their expression of three cell adhesion molecules which may account, in part, for their metastatic nature. They have lost expression of E-cadherin and the integrin b4, two adhesion molecules thought to play roles in maintaining a "normal" phenotype (Fig 5). They have upregulated expression of $\alpha v\beta 3$. Of the 13 breast cancer cell lines that we have examined to date, MDA-MB-435 and MDA-MB-157 are the only cell lines which clearly express $\alpha v\beta 3$. While we have observed occasional expression of $\alpha v\beta 3$ in tumor specimens, it has not been a consistanc characteristic of breast tumors.

Immunohistochemistry of breast tumor tissue. We have evaluated a few more tumor tissues this past year for integrin expression as well as cell cycle proteins (cell cycle kinases, cylins A and D). The results for the integrin profile on tumor cells was the same on the new specimens as we had observed previously. Staining with antibodies to the cell cycle proteins (cdks and cyclins) was not particularly illuminating

TABLE I

Cells	Percentage positive cells				Changes in kinase activity	
	$\alpha 5\beta 1$	$\alpha v\beta 3$	$\alpha v\beta 5$	Neg. con.	cdc2	cyclin A
HMEC	95.3	0.8	1.2	1.2	none	increase
T47D	50.4	5.2	5.2	5.2	decrease	increase
Hs578T	96.0	9.6	1.0	1.0	none	none
MDA-MB-453	1.9	1.4	1.5	1.5	increase	none
MDA-MB-157	19.3	34.3	2.0	2.0	increase	decrease
DU4475	1.8	7.0	1.4	1.4	none	increase
MDA-MB-361	22.2	3.8	5.9	5.9	increase	none
MCF-7	unk	10.4	10.2	10.2	none	increase

Table I: Summary of RGD peptides effect on cdc2 and cyclin A associated kinase activity in normal human mammary epithelial cells (HMEC) and breast carcinoma cell lines. Percentage positive cells is from FACS analysis of integrin expression after setting a gate to exclude cells stained with non-specific isotype-matched control antibodies.
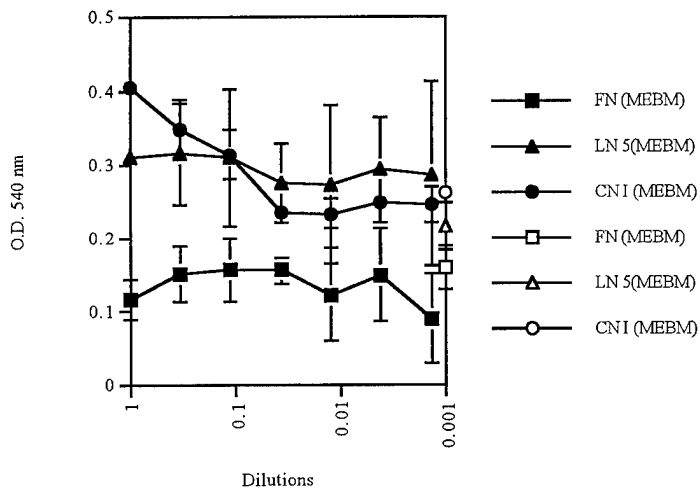

Fig. 1. Stimulation of HMEC cell proliferation by ECM proteins. HMEC cells were synchronized by culture in the absence of growth factors for 36 h. Cells were then replated onto 96 well plates coated with ECM proteins diluted 3-fold down the plate. After 3 days growth under growth factor-free conditons, the cells were fixed and stained with crystal violet. The bound dye was eluted with SDS and the absorbance of each well was measured at 540 nm. The data are the average of 3 wells with the corresponding standard deviation. Open symbols are cell adhesion to uncoated wells.
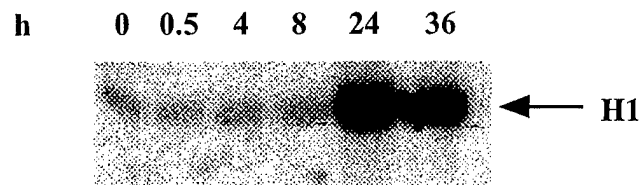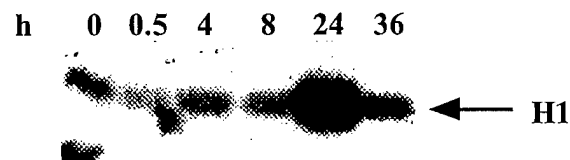
2A.   **cdc2 kinase activity, GF (+)**

h   0   0.5   4   8   24   36

⟵ H1

2B.   **cdc2 kinase activity, GF (+)**
                 β1 clustered

h   0   0.5   4   8   24   36

⟵ H1

Fig. 2. Effect of integrin β1 clustering on cdc2 kinase activity upon release from growth arrest.

HMEC were synchronized by growth factor starvation. Cells were stimulated to grow by the addition of growth factors either alone (2A) or in conjunction with clustering of the β1 integrins (2B). Cell lysates were prepared at the indicated times and assayed for cdc2 kinase activity.

14

h 0 6 8 12 16

beta-1
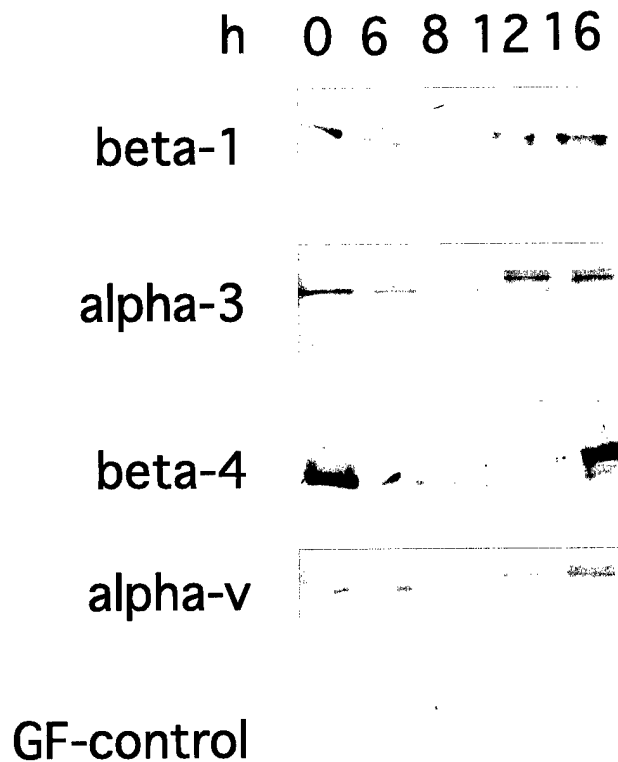
alpha-3

beta-4

alpha-v

GF-control

Fig. 3. Time course of Rb phosphorylation induced by integrin activation.

HMEC cells were synchronized by removal of all growth factors from the serum-free media for 36 h. Integrins were clustered with subunit specific monoclonal antibodies as described in Methods. At the indicated times after addition of the cross-linking secondary antibodies, lysates were prepared. Equivalent amounts of protein were seperated by SDS-PAGE, transferred to Immobilon membrane and probed with an antibody for the Rb protein. Phosphorylation of Rb is indicated by a shift to an apparent higher molecular weight.
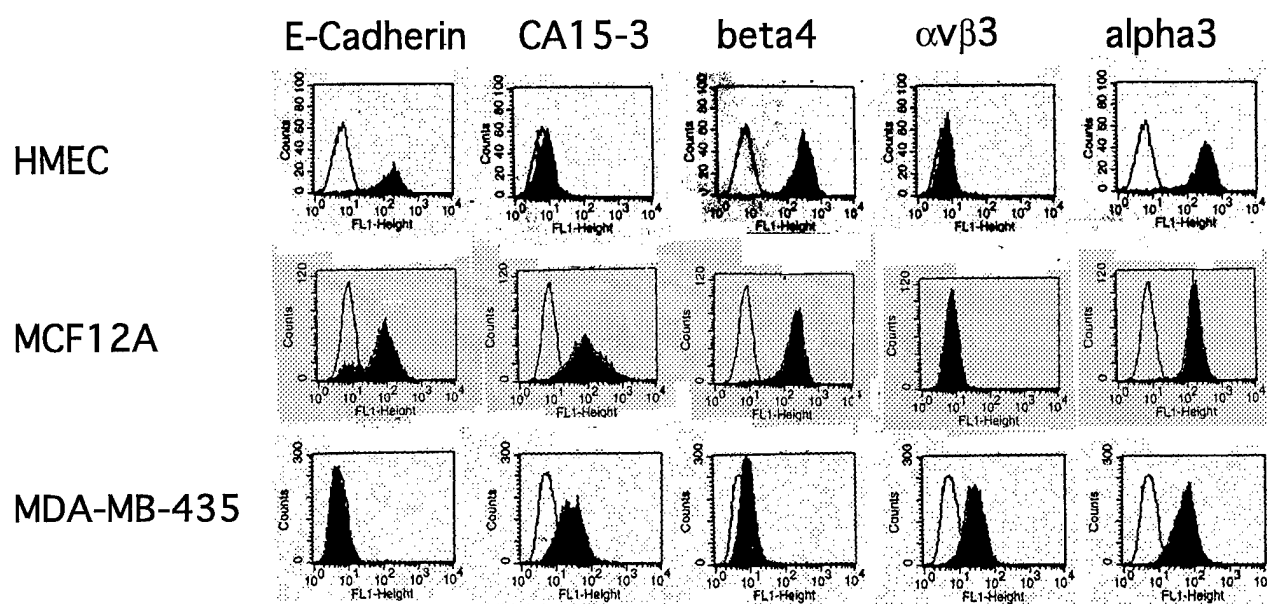
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Fig. 4. Cell surface markers define normal HMEC cells as basal cells, MCF-12A as luminal cells and MCA-MB-435 as ductal carcinoma cells.

Cell surface expression of E-cadherin, CA15-3, integrin $\beta4$, integrin $\alpha v\beta3$, and integrin $\alpha3$ was determined by FACS analysis using monoclonal antibodies and FITC-conjugated secondary antibodies. Expression of each cell surface protein is indicated by the filled area and is shown in comparison to non-specific monoclonal antibodies (open trace).
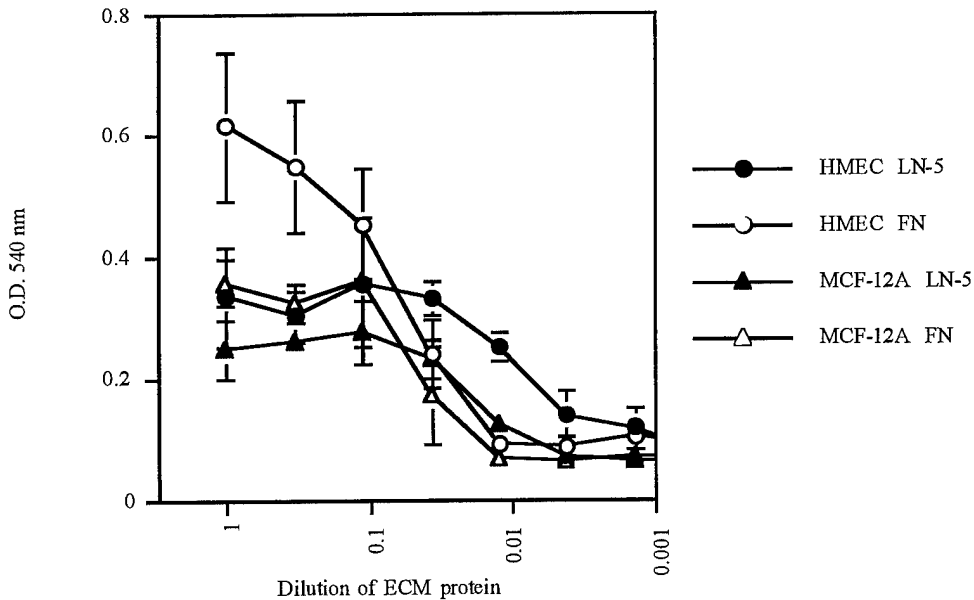
16

Fig. 5. Adhesion of MCF12A and HMEC to laminin5(LN-5) or fibronectin (FN).

HMEC (circles) or MCF12A (triangles) were adhered to decreasing amounts (3 fold dilutions) of either laminin -5 (closed symbols) or fibro- nectin (open symbols) for 30 min at 37°C. The start- ing concentration of fibro- nectin was 30 μg/ml and the starting concentration of the LN-5 capturing antibody, C2-5, was 50 μg/ml. Plates were analyzed as described in Methods.
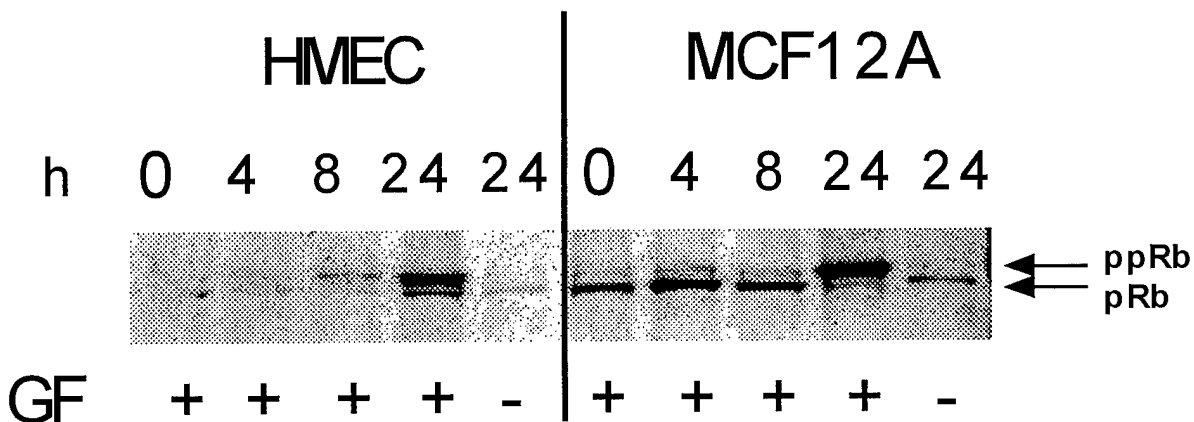

Fig. 6. Time course of retinoblastoma protein (pRb) phosphorylation after release from growth arrest.

HMEC or MCF-12A cells were synchronized by culturing under growth factor-free conditions for 48 h. Cells were refed with media containing the full complement of normal growth factor supplements at time 0. Cells were lysed at the indicated time points and equivalent amounts of protein were seperated by SDS-PAGE, transferred to PVDF membrane, and probed with a monoclonal antibody to Rb. Cells were also maintained under growth factor free (GF (-)) conditions as a negative control. Phosphorylation of Rb (ppRb) is indicated by an increase in apparent molecular weight.

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III. Conclusions:

During this past year, we have focused mainly on Aim 1 of the revised Statement of Work. We have taken a fairly broad approach and tried to address this question by looking at a variety of ligands (laminin5, fibronectin, collagen I, RGD polymers) and signaling through broad families of integrins ($\beta1$ and αv) as well as specific integrins that may be more relevant to mammary epithelial cells, $\alpha3\beta1$ and $\alpha6\beta4$.

Our results indicate that multiple integrins ($\alpha3$, $\beta4$, αv-Fig 3) are capable of stimulating cell proliferation. Growth stimulation by ECM proteins (Fig 1) suggests that receptors for collagen I ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$), possibly laminin5 ($\alpha3\beta1$, $\alpha6\beta4$, $\alpha6\beta1$), but not fibronectin ($\alpha5\beta1$, $\alpha4\beta1$, $\alpha v\beta1$, $\alpha v\beta6$) are capable of conferrring growth stimulating signals in HMEC. These results are surprising since HMEC express $\alpha5\beta1$ which has been shown to stimulate cell growth in other epithelial cell types. However, there are several parameters (fibronectin concentration, soluble versus surface-adsorbed, role of growth factors) that need to be clarified before making any definite conclusions. These results also need to be corroborated by other means such as thymidine incorporation into DNA, Rb phosphorylation, and/or cdk activities. We plan to complete these experiments within the next couple of months.

For the moment, we are using the phosphorylation of Rb as our primary assay to evaluate the role of integrins in regulating cell proliferation. This assay permits us to process our samples in a more timely fashion than our assays for cdk activities. We are also starting with a more uniform cell population because they have been synchronized by growth factor deprivation.

Our data on the expression of the CA15-3 antigen by MCF-12A cells but not normal HMEC suggests that the MCF-12A cell line should serve as useful model for the study of cell growth regulation on luminal mammary epithelial cells. Kao, C-Y et al (1995) reported that basal or luminal cells could be isolated from normal breast tissue by altering the media composition suggesting that proliferation may be regulated via different mechanisms in these two cell types. Since most breast carcinomas arise from luminal epithelial cells, we feel that it is important to study the regulation of cell proliferation in luminal and not basal mammary epithelial cells. During this next year, we will expand our use of MCF-12A cells to study the role of integrins in cell cycle regulation. We will continue our efforts to optimize conditions for the integrin clustering experiments. We will also test these cells in the ECM-cell growth assays.

Even though MCF12A cells have many characteristics of normal luminal mammary epithelial cells, they are an immortilized cell line which indicates that there has been some alteration of the growth regulation machinery. We will therefore explore the use of cell surface markers to sort the luminal cells from the basal cells using either a fluroescence activated cell sorter or antibodies coupled to magnetic beads. We will use the CA15-3 antigen as our marker for luminal cells. We have several vials of cells isolated from normal breast tissue stored in liquid nitrogen which should be suitable for these experiments.

Initially we will determine by double labeling FACS whether or not the CA15-3 positive cells also express either E-cadherin or the integrin β4. We will also utilize the culture conditions described by Kao, C-Y et al. (1995) to promote the cultivation of the luminal cell population. If these efforts are successful, we will use these cells for future experiments.

Most of our efforts this next year will be focused on Aim 2 to determine the mechanisms of integrin-mediated cell cycle regulation Several investigators have demonstrated that integrin occupancy and clustering mediates the clustering and activation of growth factor receptors (Miyamoto et al., 1996, Plopper et al., 1995). These studies were mostly conducted in fibroblasts. We will look for similar mechanisms in mammary epithelial cells with regards to receptors for epidermal growth factor, platelet derived growth factor, and basic fibroblast growth factor. We will look at several signaling mechanisms that have been linked to integrins such as tyrosine phosphorylation, focal adhesion kinase (FAK), and mitogen-activated protein (MAP) kinase. Since the synthesis of cyclin D is tightly regulated during G1, we will also look at the time course of expression in response to integrin action. (Zhu et al., 1996).

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V. Appendices

A. Bibliography of publication and meeting extracts.

NONE

B. List of personnel:

1. Richard Tamura, Ph.D., Principal Investigator
2. Elizabeth Wayner, Ph.D,. Principal Investigator
3. Wendy Pabich, Research technician I

C. Notification of appointment of Dr. Richard Tamura as co-principal investigator. Please see attached pages.

REPLY TO
ATTENTION OF:

May 5, 1997

Special Projects Branch/LJW/wac

SUBJECT: Grant No. DAMD17-94-J-4303
           Modification P70004

Ms. Sharon Sumpter
Seattle Biomedical Research Institute
4 Nickerson Street, Suite 200
Seattle, Washington 98109-1651

Dear Ms. Sumpter:

    Enclosed are fully executed copies of the above subject grant modification for your records and the Principal Investigator's.

    If you have any questions concerning this matter, you may contact L. Joan Wilson, Contract Specialist, at (301) 619-2387 or the undersigned at (301) 619-2034.
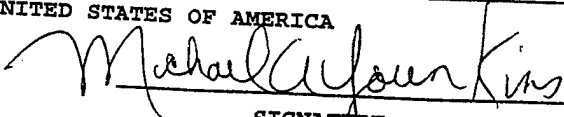
                         Sincerely,

                         Wendy A. Cockerham
                         Procurement Technician

Enclosures

GRANT AGREEMENT

GRANT NO: DAMD17-94-J-4303 Modification P70004	EFFECTIVE DATE See Grants Officer Signature Date Below	GRANT AMOUNT $561,026.40	Page 1 of 1 L. Joan WIlson 301-619-2387

PROJECT TITLE: Mechanism of Integrin-Mediated Growth Control in Normal, Transformed, and Neoplastic Breast Cells

PERFORMANCE PERIOD: September 30, 1994-October 29, 1999 (Research ends September 29, 1999)	CFDA 12.420
	PRINCIPAL INVESTIGATOR: Richard Tamura, Ph.D & Elizabeth Wayner, Ph.D.

AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-V 820 Chandler St. Fort Detrick Maryland 21702-5014	PAYMENTS WILL BE MADE BY: Army Vendor Pay DFAS-SA/FPA 500 McCullough Ave. San Antonio, TX 78215-2100 EFT:T
AWARDED TO: Seattle Biomedical Research Institute 4 Nickerson Street, Suite 200 Seattle, WA 98109-1651	REMIT PAYMENT TO: SAME AS AWARDED TO BLOCK

ACCOUNTING AND APPROPRIATION DATA: N/A

SCOPE OF WORK:

1. THE PAYING OFFICE NAME AND ADDRESS IS HEREBY CHANGED TO THAT SPECIFIED IN THE "PAYMENT WILL BE MADE BY" BLOCK ABOVE.

2. THE PRINCIPAL INVESTIGATOR, CITED ON THE FACE PAGE OF THE GRANT, IS HEREBY CHANGED TO READ AS STATED ABOVE, INDICATING THAT DR. ELIZABETH WAYNER AND DR. RICHARD TAMURA WILL ACT AS CO-PRINCIPAL INVESTIGATORS.

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ACCEPTED BY: _____ SIGNATURE	**UNITED STATES OF AMERICA** _[signature]_ SIGNATURE
NAME AND TITLE DATE 25	NAME AND TITLE MICHAEL A. YOUNKINS GRANTS OFFICER DATE 5/2/97

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